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PHOSPHOLIPID METABOLISM IN THE BROWN ALGA, *FUCUS SERRATUS*

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Abstract—The metabolism of phospholipids in the brown alga, *Fucus serratus* was studied. The major phospholipids of this alga are phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, cardiolipin and phosphatidylcholine. When the time-course of labelling of the lipids from [^{32}P] orthophosphate was studied, total labelling was approximately linear for 8 hr. All the major classes of phospholipid were labelled. The extent and pattern of labelling were not affected by the presence of proteins synthesis inhibitors phosphatidic acid was highly labelled at short time intervals. Phosphatidylcholine was relatively poorly labelled. The extent and pattern of labelling were not affected by the presence of protein synthesis inhibitors indicating that the enzymes involved in phospholipid synthesis have a rather slow turnover. Incorporation of radioactivity into phosphatidylglycerol was stimulated significantly by light.

INTRODUCTION

The brown algae represent a very important and prolific class of organisms. In spite of this, relatively little is known about their lipid composition in comparison to higher plants or Chlorophytae. Lipids constitute about 3% of the dry wt of *Fucus* sp. [1] with large amounts of the three characteristic plant glycosylglycerides, diacylsulphoquinovosylglycerol (SQDG), diacylgalactosylglycerol (MGDG) and diacylgalabiosylglycerol (DGDG) [2]. The major phospholipid is phosphatidylethanolamine with smaller amounts of phosphatidylglycerol, phosphatidylinositol, cardiolipin and phosphatidylcholine [3]. A number of other minor acyl lipids have also been identified, some of which have unusual or unique structures [4, 5]. The Phaeophyta are also very rich in polyunsaturated fatty acids with large amounts of octadecatetraenoic, eicosatetraenoic and dodecapentaenoic acids [6].

To date there have been no reports of experiments on lipid metabolism in Phaeophytae. As part of a study of the effect of the environment on lipid metabolism and photosynthesis in *Fucus serratus*, we have examined some features of phospholipid synthesis. The results of these experiments are now reported.

RESULTS AND DISCUSSION

To ensure quantitative extraction of phospholipids from *F. serratus*, together with minimal contamination from epiphytic micro-organisms, a number of methods for washing and extracting the algae were tested. An effective way of removing most of the micro-organisms (as judged by microscopic examination and bacterial culture on high-salt agar plates) was found to be by washing with a dilute detergent solution. This treatment (under the conditions described in the Experimental) did not result in any impairment of [^{32}P] orthophosphate uptake or incorporation into the phospholipids of *F. serratus*. Of several extraction methods used, it was found that the high ionic method of Garbus *et al.* [7], together with a pretreatment with boiling *iso*-propanol to inactivate lipid-metabolizing enzymes [8], was the most effective and gave the most consistent results.

Quantitative analysis and identification of endogenous phospholipids was carried out (see Experimental) and, in agreement with previous workers, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylcholine and cardiolipin were found to be major components with traces of phosphatidylserine and phosphatidic acid (Table 1). Phosphatidylethanolamine was the major phospholipid. These results were also broadly in agreement with the results of Liem and Laur [9, 10] except that neither Pohl [3] nor ourselves found very large amounts of phosphatidylcholine. The lipid composition of the Golgi membrane of *F. serratus* has also been reported [11] but in this case the identity of the components was based on their chromatographic mobility relative to lipid markers and no degradative studies were carried out.

The time-course of labelling was studied and it will be seen that incorporation of radioactivity from [^{32}P] orthophosphate was approximately linear with time until *ca.* 8 hr after which the rate decreased somewhat (Fig. 1a). It was considered possible that the slowing of radio-incorporation at longer incubation times was due to a deterioration of the algal preparations.

Table 1. Time-course of the labelling of phospholipids from [^{32}P] orthophosphate in *F. serratus*

Time (hr)	No. of experiments	Distribution of radioactivity (%[^{32}P] lipids)						
		PA	CL	PG	PE	PC	PI	Other
1	3	13.6 ± 1.8	10.4 ± 2.0	6.3 ± 0.8	46.2 ± 0.7	5.5 ± 0.4	14.0 ± 1.0	4.0 ± 0.5
2	4	10.3 ± 1.9	12.5 ± 1.7	7.5 ± 1.3	44.5 ± 4.2	5.6 ± 2.4	14.2 ± 2.0	5.4 ± 1.0
4	9	6.9 ± 1.0	12.2 ± 2.4	7.0 ± 1.1	50.0 ± 2.6	5.4 ± 0.8	15.4 ± 2.0	3.1 ± 0.3
8	4	4.0 ± 1.4	13.1 ± 2.0	6.6 ± 1.2	51.7 ± 2.8	3.4 ± 0.5	18.1 ± 2.6	3.1 ± 0.3
16	1	3.3	9.9	8.3	49.3	3.6	21.3	3.9
24	6	2.2 ± 0.2	11.4 ± 1.2	8.1 ± 0.7	54.7 ± 1.6	4.9 ± 0.8	17.0 ± 0.8	1.7 ± 0.2
Endogenous phospholipid	4	tr.	18.4 ± 3.7	13.0 ± 2.3	36.0 ± 5.2	16.2 ± 2.4	14.4 ± 3.8	2.0 ± 1.7

Incubations were carried out in phosphate-free ASP-6 medium at 15° with *ca.* 1000 lx illumination. Results = means ± s.e.m.

Abbreviations: PA, phosphatidic acid; CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; tr. = trace (< 0.1%). Endogenous levels are expressed as phospholipid phosphorus as a percentage of the total phospholipid phosphorus.

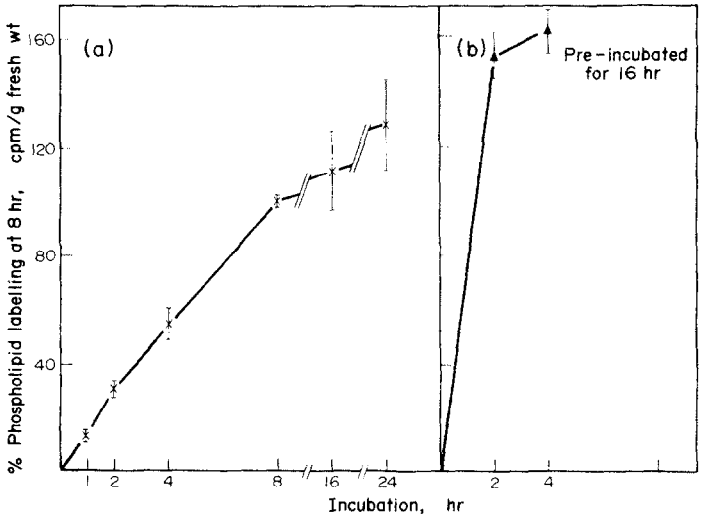

Fig. 1. Time-course of the incorporation of radioactivity from [^{32}P] orthophosphate into the lipids of *F. serratus*. Data from four separate experiments are shown with the means ± s.d. indicated. For the pre-incubations (b), *Fucus* samples were incubated in ASP-6 medium for 16 hr and then transferred to phosphate-free ASP-6 medium containing radiolabel. Other samples (a) were incubated directly in phosphate-free ASP-6 medium containing radiolabel. The same volume of carrier-free [^{32}P] orthophosphate was added in all cases (0.8–4 μCi/g fr. wt). Counts were adjusted to allow for the decay of the isotope in order to compare individual experiments.

Accordingly, pre-incubation of tissue samples for 24 hr was carried out before the addition of [^{32}P] orthophosphate. It can be seen that the pre-incubation did not reduce the rate of phospholipid labelling. In fact, labelling was more rapid and maximum incorporation was obtained in a shorter time (Fig. 1b). The pattern of phospholipid labelling was unchanged by pre-incubation of the algae (data not shown). We conclude, therefore, that the reduction in labelling rates after long incubation times are due to exhaustion of radiolabel in the medium together with its equilibration with the endogenous phosphate pool(s). This conclusion was supported by an increase in the rates of labelling on addition of more [^{32}P] orthophosphate and by the small amount of [^{32}P] orthophosphate remaining in the medium (unpublished data).

The [^{32}P] phospholipids were separated by one- and two-dimensional TLC in order to determine the distribution of label at different incubation times. Results for several time points are shown in Table 1. All of the major phospholipids were appreciably

labelled but there were only small amounts of radioactivity detected in phosphatidic acid (except at short time intervals) and trace amounts (< 1%) in phosphatidylserine. At all time intervals in the period 1–24 hr phosphatidylethanolamine contained the highest amount of radiolabel. The relative labelling of phosphatidic acid was significantly greater at short time intervals, indicating a high turnover of this phospholipid. This was not unexpected since phosphatidic acid is known to be an important intermediate in phospholipid synthesis [12]. Although phosphatidylcholine is rapidly labelled in many higher plant and algal tissues [13], it was not very highly labelled in *F. serratus*. The proportion of label in phosphatidylinositol and that in cardiolipin and phosphatidylglycerol, which are metabolically related [12], remained approximately constant with time. The major phospholipid, phosphatidylethanolamine, contained an increasing amount of radiolabel with time up to 8 hr of incubation. In terms of their contribution to the total mass of phospholipid in *F. serratus*, phosphatidylethanolamine had a higher specific radioactivity than the total pool at all times while phosphatidic acid had a higher specific radioactivity after 1 or 2 hr incubation. Cardiolipin, phosphatidylglycerol and especially phosphatidylcholine, were labelled at lower rates than the total phospholipid pool.

The pattern of phospholipid labelling was not affected by pre-incubation of tissue for 24 hr or by the use of tissue which had been kept under laboratory conditions (see Experimental) for up to 4 weeks.

The high and rapid rates of phospholipid labelling which we had found in *F. serratus*, together with its eukaryotic nature, led us to consider whether the incorporation of radioactivity into phospholipids could be prevented by the use of selective inhibitors of protein synthesis. Cycloheximide and chloramphenicol were used as inhibitors of nuclear and plastid/mitochondrial coded protein synthesis respectively [14]. They were used at concentrations which are known to be effective against other organisms including higher plants [15] and algae [16]. As can be seen in Table 2, neither of the inhibitors affected the rate of radiolabelling of phospholipids. The pattern of labelling also remained the same as for control tissues (results not shown). The lack of inhibition of phospholipid labelling by cycloheximide or chloramphenicol could have been due to a number of reasons. These included a lack of penetration of the inhibitors, the non-sensitivity of *Fucus* protein synthesis to the compounds (at least at the concentrations used) or to a slow turnover of the enzymes involved in phospholipid biosynthesis. To exclude the first two reasons, we also tested the effect of cycloheximide and chloramphenicol on protein synthesis. The normal methods for measuring protein synthesis involve the incorporation of radiolabelled amino acids and the precipitation and washing of the labelled material with such chemicals as acids and/or organic solvents. Under most of these conditions, complex carbohydrates precipitate and their presence in high amounts in *F. serratus* forced us to use a different technique. We relied on heat-denaturation of proteins under conditions where polysaccharides such as alginic acid or fucoidin are soluble and could be removed. Control experiments showed that the soluble material after boiling did not contain any significant radioactivity in high MW compounds, so we feel that the precipitation of protein by heat treatment was effective. In addition, removal of particulate debris by filtration gave the same pattern of results as the normal procedure (see Experimental) although with a reduction (*ca.* 20%) in the total amount of radioactivity incorporated from [^{14}C] proline. We do not, therefore, believe that non-protein cell wall components contribute significantly to the total incorporation of radioactivity. In Table 2 it can be seen that inhibitors (at the concentrations used when measuring phospholipid synthesis) resulted in decreased incorporation of radioactivity from [^{14}C] proline or [^{14}C] leucine. Cycloheximide resulted in *ca.* a 50% reduction in the labelling of the heat-denatured proteins from either precursor within 24 hr.

Table 2. Effect of protein synthesis inhibitors on protein and phospholipid labelling in *F. serratus*

Inhibitor	Concentration (μg/ml)	Protein labelling (% control) Time (hr)	[^{14}C] Proline precursor	[^{14}C] Leucine precursor	Phospholipid labelling (% control)
Cycloheximide	10	4	85 ± 22	64 ± 9	102 ± 10
	10	24	65 ± 11	—	93 ± 9
	100	4	—	48 ± 16	—
	100	24	44 ± 2	56 ± 27	—
Chloramphenicol	50	4	44 ± 11	44 ± 20	86 ± 7
	50	24	2 ± 1	11 ± 2	103 ± 9
	100	4	10 ± 5	4 ± 2	—
	1000	24	5 ± 2	9 ± 3	—

Figures represent means ± s.d. where $n = 3$ for protein and phospholipid labelling. Incubations with [^{14}C] proline were carried out in ASP-6 medium. Incubations with [^{14}C] leucine were carried out in sterilized sea water and those with [^{32}P] orthophosphate in both media. No significant increase in epiphytic micro-organisms was observed during the incubation period. Radiolabelling was calculated on a fr. wt basis.

Table 3. Effect of light on the incorporation of [^{32}P] orthophosphate into the phospholipids of *F. serratus*

	Total labelling (cpm/g fr. wt)	Distribution of label (% total lipid)					
		PA	CL	PG	PE	PC	PI*
Light	62 202/153 048	1.5 ± 0.3	10.8 ± 2.4	11.6 ± 0.8	52.1 ± 8.6	5.1 ± 0.6	18.9 ± 3.3
Dark	68 362/135 233	1.4 ± 0.4	9.8 ± 0.6	10.3 ± 1.0†	54.3 ± 5.9	4.9 ± 1.9	19.3 ± 2.9

Results = means ± s.d. ($n = 4$). For abbreviations see the legend to Table 1. Incubations were carried out in phosphate-free ASP-6 medium with the addition of carrier-free [^{33}P] orthophosphate for 4 hr at 15°. Light-treated samples were exposed to *ca.* 1000 lx.

*The PI band also contained small amounts of other radiolabelled phospholipids, e.g. phosphatidylserine.

†Significantly different ($P < 0.05$) by Student's t test for paired samples.

In contrast, chloramphenicol caused a much higher inhibition of labelling. It was found that the uptake of amino acids into the algae was reduced by chloramphenicol and this accounted for the large inhibition of labelling. Such unspecific effects on transport have been reported before for chloramphenicol [14]. A comparison of the inhibition of protein synthesis by different amounts of cycloheximide or chloramphenicol (Table 2), shows that the concentrations used to test phospholipid synthesis were suitable. We conclude, therefore, that the enzymes involved in phospholipid synthesis in *F. serratus* have a slow turnover.

Light is known to play an important role in the synthesis of membrane lipid components in photosynthetic tissues [17, 18]. Its effect on the incorporation of radioactivity from [^{32}P] orthophosphate into lipids is shown in Table 3. Under the experimental conditions, light had relatively little effect on the rate or distribution of radioactivity between different lipid classes. There was a small increase in the relative labelling of phosphatidylglycerol in the presence of light in keeping with this lipid's role as the major phospholipid component of plastid membranes [18, 19] and with the known effect of light on its metabolism in higher plants [17]. The rate of synthesis of a typical non-plastid phospholipid, such as phosphatidylethanolamine, was unaffected by light (Table 3) which we interpret as meaning that the supply of necessary cofactors for phospholipid synthesis is not dependent on photosynthesis during the incubation period. Under such conditions, the labelling of typical plastid lipids such as the glycosylglycerides and of the polyunsaturated fatty acids from [^{14}C] acetate was altered by the presence of light (unpublished results).

These data show that the phospholipids of *F. serratus* are synthesized at high rates by enzymes which are themselves rather slowly turned over. The rapid metabolism of typical non-plastid phospholipids may be due to their role in Golgi body formation and polysaccharide secretion in *F. serratus* [11], although proof for such a function must await further experimentation.

EXPERIMENTAL

Tissue. Healthy samples of *F. serratus* were collected from Lavernock Point in the Severn Estuary in the period Nov.–Jan. The tissue was washed with sterilized sea water (collected from the same location) and kept in sterilized sea water at 4° with aeration under an illumination of *ca* 1000 lx. Control expts showed that the viability of the algae as measured by photosynthetic rates, lipid metabolism and (microscopic) appearance remained good for at least 4 weeks although the tissue was generally used within 2 weeks of collection.

Assessment of microbial contamination. The presence of epiphytic organisms on the *Fucus* fronds was assessed by examination by light microscopy. The major class of epiphytic organisms was bacteria. These were also cultured using a high-salt medium [20] in order to assess the efficiency of various washing procedures for removing microbial contamination. Of the various methods used, it was found that 0.5% (w/v) Triton X-100 in ASP-6 medium [21] was the most effective procedure for removing epiphytic organisms without affecting photosynthesis or lipid metabolism. Examination of such fronds by microscopy or by culture indicated that at least 95% of the bacteria were removed by such treatment. In addition, *F. serratus* was examined by electron microscopy using an araldite-embedding medium and a Philips 400 microscope.

Incubation conditions. Pieces of tissue (*ca.* 0.1 g each) were cut from the frond tips, rinsed briefly in 0.5% Triton X-100 in ASP-6 medium and the detergent removed by successive washes in ASP-6 medium [21]. The tissue was incubated at *ca.* 0.2 g/ml Pi-free ASP-6 medium containing [^{32}P] Pi (carrier-free; Radiochemical Centre, Amersham, U.K.) at 15° in a shaking water-bath. Illumination (when required) was *ca* 1000 lx.

Lipid extraction, identification and analysis. At the end of the incubation period, the tissue was removed, thoroughly washed and heated in *iso*-PrOH at 80° for 30 min in sealed tubes. The tissue was then ground in a pestle and mortar and extracted using the procedure of Garbus *et al.* [7] except that *iso*-PrOH replaced MeOH. The lower phase was removed, washed with synthetic upper phase and aliquots removed for the measurement of total phospholipid labelling. The lipid extract was separated by chromatography on Si gel G TLC plates using a 2-dimensional system with $CHCl_3$–MeOH–H_2O (65:25:4) in the first dimension and $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (10:4:2:2:1) in the second dimension or using a one-dimensional system with $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7). Phosphatidylinositol was separated from phosphatidylserine by TLC on 1 mM Na_2CO_3–Si gel H plates using $CHCl_3$–MeOH–HOAc–H_2O (25:15:4:2) as solvent. Routine identification of radioactive components was with authentic standards. In-

dividual lipids were identified by the use of differential sprays and alkaline and acidic degradation [18, 22]. Quantification of phospholipid mass was by phosphate determination or from fatty acid methyl ester measurement [18, 22]. Radioactive compounds were revealed by spark-chamber autoradiography and individual phospholipids were removed for scintillation counting [23]. Correction for quenching was by the channels ratio method.

Measurement of protein synthesis. Protein synthesis was measured by the incorporation of radioactivity from [U-^{14}C]proline (250 μCi/μmol) or [U-^{14}C]leucine (10 mCi/mmol; Radiochemical Centre, Amersham, U.K.). At the end of incubation the algal samples were rinsed well and heated at 100° in sealed tubes in 1 ml H_2O for 1 hr. After cooling, the samples were homogenized, an additional 4 ml H_2O added and the homogenate heated at 100° in sealed tubes for 4 hr. After this time, the insoluble material was centrifuged down and the ppt. rinsed (×3) with EtOH. The insoluble material was finally solubilized with Soluene® (Packard), decolourized and counted [23].

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